

ENZYME KINETICS

INTRODUCTION

The study of reaction rates catalyzed by enzymes and the factors affecting them is generally referred to as enzyme kinetics. The basic components of an enzyme catalyzed reaction are substrate, enzyme and product. These are diagrammed in Figure 1.

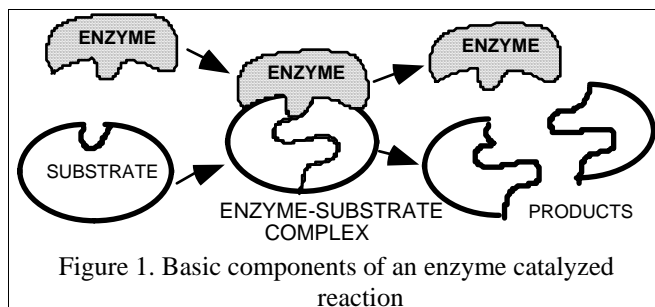


Figure 1. Basic components of an enzyme catalyzed reaction

In this lab you are going to study the kinetics of an enzyme called acid phosphatase (abbreviated: AcPase). It occurs in most living things and is generally associated with the lysosomes within cells. AcPase acts on certain molecules that carry phosphate groups by cleaving off the phosphate group (Fig. 2). The liberated phosphate returns to the phosphate pool of the cell.

We choose a substrate that favorably lends itself to kinetic study. AcPase acts on Paranitrophenol Phosphate (PNPP) by removing its Phosphate,

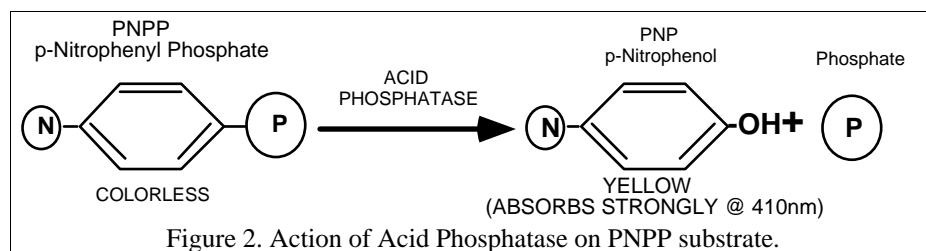


Figure 2. Action of Acid Phosphatase on PNPP substrate.

leaving Paranitrophenol (Fig. 2). What makes this reaction ideal for study is that the product is colored, whereas the substrate is not. Thus, if you added AcPase to a solution of PNPP, the solution would initially be clear, but become more and more yellow as the reaction progresses.

The laboratory procedure for studying an enzyme is called an assay and the assay for Acid Phosphatase is diagrammed in Figure 3. Note the key elements: substrate; buffer; temperature; time; and concentration of product. The fact that PNP (yellow product) absorbs strongly in 410 nm of light, allows for accurate measurement with the spectrophotometer and subsequent estimation of concentration on a standard curve.

You are going to study the Acid Phosphatase reaction in four segments. In each segment one of the key elements will be varied, while the others are kept constant. The first segment involves studying the reaction over time (Fig. 5); the second, third, and fourth involve variation in substrate concentration (Fig. 6), pH (Fig. 7), and temperature (Fig. 8) respectively.

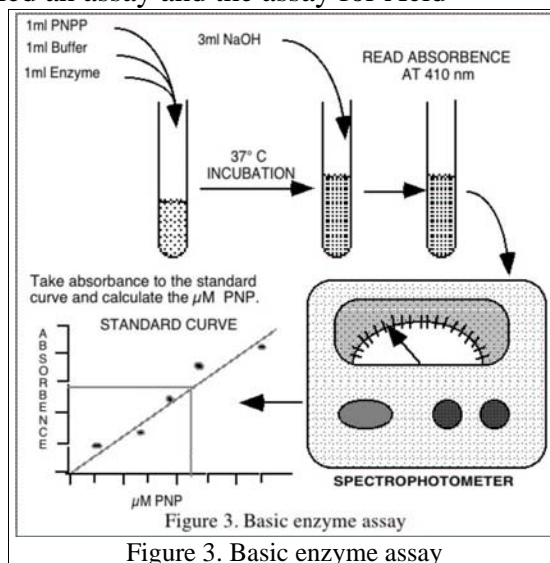


Figure 3. Basic enzyme assay

A. STANDARD CURVE

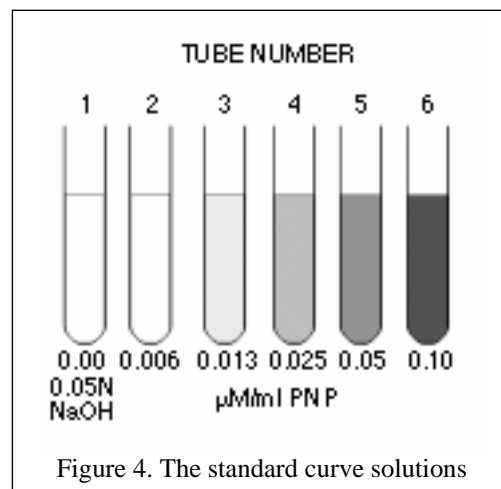
In order to determine the amount of product (PNP) formed during the reactions, you will need a standard curve.

EQUIPMENT

- 6 test tubes in a rack
- 2 matched cuvettes
- 2 - 10 ml pipettes
- 10 ml of 0.10mM PNP stock solution
- 30 ml 0.05M NaOH

MAKE THE DILUTIONS FOR THE STANDARD CURVE

1. You will be provided with a 0.10mM stock solution of PNP to make dilutions from.
2. Always wear gloves when working with the PNP or PNPP solutions.
3. Be sure to use 0.05M NaOH to make your dilutions.
4. Label test tubes 1 through 6
5. Tube #1 will be 0.05M NaOH and Tube #6 will contain the stock 0.10mM PNP solution.
6. You will need 3 ml of solution to make the spectrophotometer reading.
7. Using the stock solution and the 0.05M NaOH, make a 1/2 dilution series so that tubes 2, 3, 4, & 5 will contain 5 ml of the concentrations as indicated in Figure 4.
8. Line the tubes in order in the test tube rack.



MEASURE THE ABSORBANCE OF EACH TUBE

9. Move to a spectrophotometer and ensure that it is properly warmed up.
10. Since you must use the same spectrophotometer each time you use this standard curve, record the number of your spectrophotometer in Table 1.
11. Pour the contents of tubes #1 and #2 into cuvettes.
12. Ensure the spectrophotometer is set at 410 nm.
13. Select the "A" (absorbance) mode.
14. Insert tube #1 into the spectrophotometer and press the "0 ABS/100% T" key. This is called zeroing the spectrophotometer. From now on, the tube used to zero the spectrophotometer (tube #1 in this case) will be referred to as the blank.
15. Measure the Absorbance of the other cuvette (from tube #2) and record in Table 1.
16. Pour contents of this cuvette back into tube #2, shake out the cuvette, and pour contents of tube #3 into cuvette.
17. Measure and record the Absorbance of the cuvette with its new contents. You don't have to rinse the cuvette between changes as long as you are going from a more dilute solution to a more concentrated one.

- Repeat the process so that the Absorbance of tubes #3, #4, #5, and #6 is measured and recorded.
- Dispose of all PNP (and PNPP) solutions in the special waste container provided.

ANALYSIS OF STANDARD CURVE DATA

- Make a spreadsheet in *Excel* that is similar to Table 1 and enter your absorbance data from Table 1.
- In *Excel* construct a Standard Curve Graph by plotting absorbance (X axis) against the amount of PNP present (Y axis). See Figure 3.
- Make sure that your standard curve has a trend line so that you can calculate concentrations in Parts B-E.
- You must use the same spectrophotometer each time you use this standard curve.

Tube #	Abs.	mM PNP
1		0
2		0.006
3		0.013
4		0.025
5		0.05
6		0.10

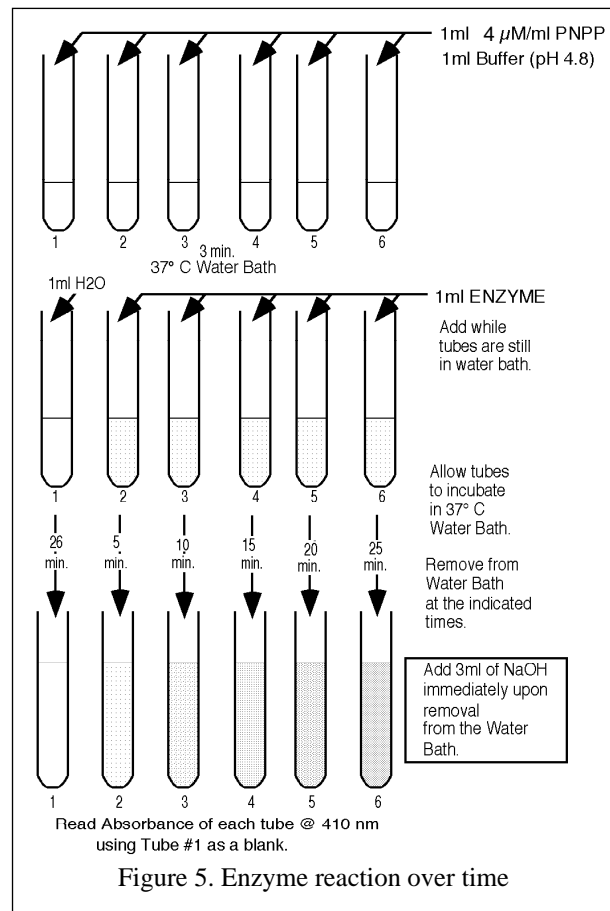
B. ENZYME REACTION OVER TIME

EQUIPMENT

- 6 Test tubes
- 4mM PNPP substrate solution
- pH 4.8 buffer
- 1ml squeeze pipettes

PROCEDURE

- Label and prepare six tubes as indicated in Figure 5.
- To each tube add 1ml of the 4mM PNPP substrate solution
- Add 1ml of buffer at pH 4.8 to each tube.
- Place all the tubes in a 37°C water bath for three minutes. This is to allow them to come to temperature before adding the enzyme solution.
- While tubes are still in the water bath, add 1ml of enzyme solution to tubes #2-#6. Be sure to note the exact time of the addition for each tube in Table 2. Add 1ml of deionized water to tube #1. This tube will serve as the blank since there is no enzyme added.
- Remove tube #2 after exactly 5 minutes, tube #3 after exactly 10 minutes, #4 after 15, #5



after 20, and tube #6 after 25 minutes incubation (Fig. 4). Immediately upon removal, add 3 ml of the 1.0M NaOH solution. This is necessary to stop the reaction and to allow proper color development of the PNP.

7. Immediately after removing tube #6, remove tube #1 and add 3 ml of 1.0M NaOH to it. Remember, this is your blank. (You will not include this blank in your graph.)
8. Locate your spectrophotometer. Remember it has to be the same one you used to make your standard curve. Be sure the spectrophotometer is turned on and had at least a 10 minute warm-up. Set the wavelength to 410 nm.
9. Pour the contents of tubes #1 and #2 into cuvettes. Use the contents of tube #1 as a blank to zero the spectrophotometer.
10. Measure the Absorbance of the cuvette with tube #2's contents and record in Table 2. Then empty the cuvette back into tube #2, shake out any drops, and add the contents of tube #3.
11. Measure the Absorbance of the cuvette with its new contents, then return them to tube #3 and fill the cuvette with tube #4. Measure the Absorbance of tubes #5 and #6 in the same way. The cuvettes don't need to be rinsed between measurements as long as you add a more concentrated solution each time.
12. Take the Absorbance readings to your Standard Curve and convert to mM PNP formed; also record in Table 2.

Table 2. Reaction rate over time					
Tube #	Time enz. added	Time NaOH added	Elapsed time	Abs.	mM PNP formed
1					
2					
3					
4					
5					
6					

C. THE EFFECT OF SUBSTRATE CONCENTRATION

In this section, the standard assay will be performed (pH 4.8; 15 min. incubation at 37°C), but substrate (PNPP) concentrations will be varied (Fig. 6).

1. Label tubes #1-#10 as indicated in Figure 6.
2. Add 1ml of buffer (pH 4.8) to all tubes.
3. Add 1ml of the indicated (Fig. 6) PNPP concentrations to the tubes. Note that they are in pairs. The reason for this is that each substrate concentration requires its own separate blank.
4. Place all tubes in a 37°C water bath for 3 minutes to allow them to come up to temperature.
5. While tubes are still in the water bath, add 1ml of enzyme solution, or 1ml of water as indicated in Figure 6. Be sure to note the exact time of the addition for each tube in Table 3. The tube receiving the water will serve as the blank for that particular substrate concentration.

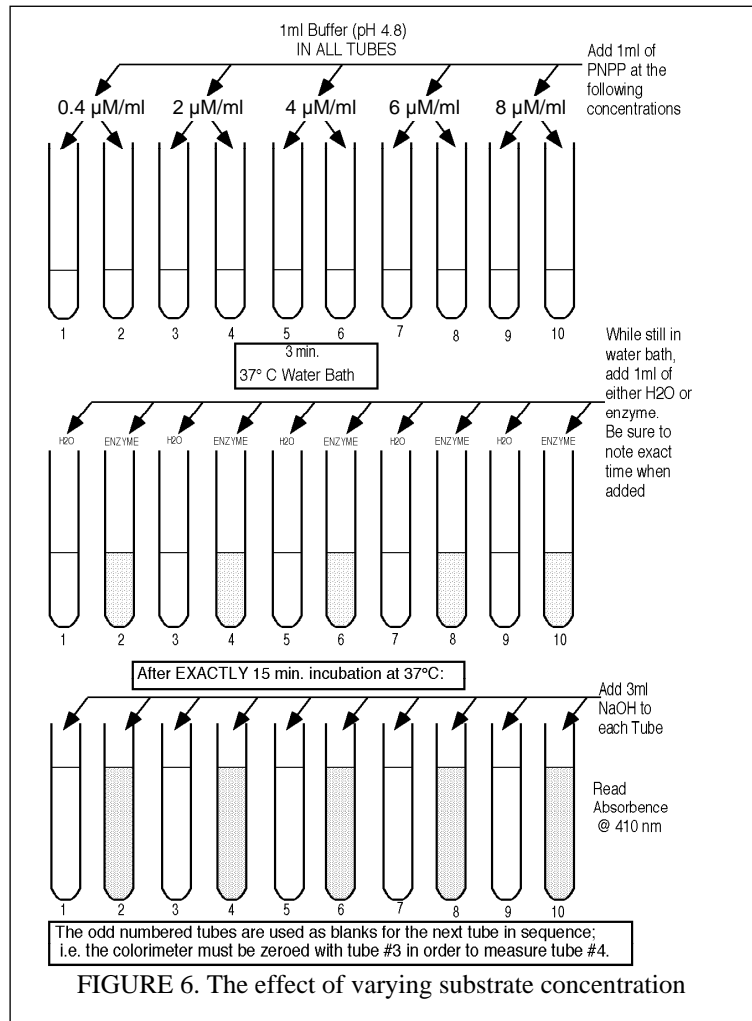
- After exactly 15 minutes incubation at 37°C, remove each tube and immediately add 3 ml of the NaOH solution.
- Be sure the spectrophotometer is turned on and had at least a 10 minute warm-up. Set the wavelength to 410 nm.
- Pour the contents of tubes #1 and #2 into cuvettes. Use the contents of tube #1 as a blank to zero the spectrophotometer.
- Measure the Absorbance of the cuvette with tube #2's contents and record in Table 3. Then empty both cuvettes back into their respective tubes, shake out any drops, and add the contents of tubes #3 and #4 to each. Be sure that tube #3 (the blank) gets poured into the cuvette which held tube #1's (also a blank) contents.

- Use the contents tube #3 as a blank and zero the spectrophotometer again. Then measure and record the Absorbance of the cuvette with tube #4's contents. Measure the Absorbance of the remaining tubes in the same way. The cuvettes don't need to be rinsed between measurements as long as you add a more concentrated solution each time and ensure that the odd numbered tubes (the blanks) always go into the same cuvette.

- Remember, the spectrophotometer must be reset to zero for each pair of tubes! In other words, every odd numbered tube will serve as a blank for the next even numbered tube.

Take the absorbance readings to your Standard Curve and convert to mM/min PNP formed and record in Table 3.

- Calculate the rate of PNP formation by dividing the mM PNP formed by 15 min. (the incubation time). Record in Table 3



Tube #	Start time	Stop time	Substrate conc.	Abs.	mM PNP formed	Rate mM/min
1			0.4mM	0		
2			0.4mM			
3			2.0mM	0		
4			2.0mM			
5			4.0mM	0		
6			4.0mM			
7			6.0mM	0		
8			6.0mM			
9			8.0mM	0		
10			8.0mM			

D. THE EFFECT OF PH

In this section, the standard assay will be performed (4mM PNPP; 15 min. incubation at 37°C), but the pH of the buffer solutions will be varied (Fig. 7).

1. Label tubes #1-#10 as indicated in Figure 7.
2. Add 1ml of the indicated buffer solutions (Fig. 7) to the tubes. Note that they are in pairs. The reason for this is that each buffer pH requires its own separate blank.
3. Add 1ml of 4mM PNPP to all tubes.
4. Place all tubes in a 37°C water bath for 3 minutes to allow them to come up to temperature.
5. While tubes are still in the water bath, add 1ml of enzyme solution, or 1ml of water as indicated in Figure 7. Be sure to note the exact time of the addition for each tube in Table 4. The tube receiving the water will serve as the blank for that particular pH.
6. After exactly 15 minutes incubation at 37°C, remove each tube and immediately add 3 ml of the NaOH solution.

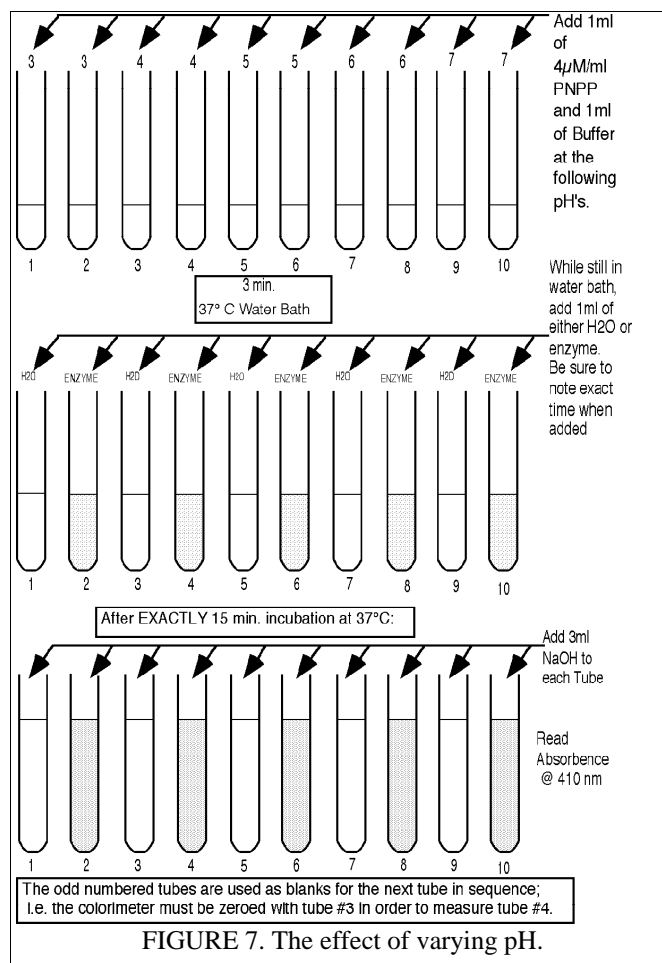


FIGURE 7. The effect of varying pH.

7. Be sure the spectrophotometer is turned on and had at least a 10 minute warm-up. Set the wavelength to 410 nm.
8. Pour the contents of tubes #1 and #2 into cuvettes. Use the contents of tube #1 as a blank to zero the spectrophotometer.
9. Measure the Absorbance of the cuvette with tube #2's contents and record in Table 4. Then empty both cuvettes back into their respective tubes, rinse with deionized water and dry with a Kimwipe, and then add the contents of tubes #3 and #4 to each.
10. Use the contents tube #3 as a blank to zero the spectrophotometer. Then measure and record the Absorbance of the cuvette with tube #4's contents. Measure the Absorbance of the remaining tubes in the same way. The cuvettes need to be rinsed between measurements because the concentration of PNP does not necessarily increase with each pair.
11. Remember, the spectrophotometer must be reset to zero for each pair of tubes! In other words, every odd numbered tube will serve as a blank for the next even numbered tube.
12. Take the absorbance readings to your Standard Curve and convert to mM/min PNP formed and record in Table 4.
13. Calculate the rate of PNP formation by dividing the mM PNP formed by 15 min. (the incubation time). Record in Table 4

Table 4. Effect of pH						
Tube #	Start time	Stop time	pH	Abs.	mM PNP formed	Rate mM/min
1			3	0		
2			3			
3			4	0		
4			4			
5			5	0		
6			5			
7			6	0		
8			6			
9			7	0		
10			7			

E. THE EFFECT OF TEMPERATURE

In this section, the standard assay will be performed (4mM PNPP; pH 4.8), but the 15 min. incubation temperature will be varied (Fig. 8).

1. Label tubes #1-#10 as indicated in Figure 8.
2. Add 1ml of the buffer solution(pH 4.8) to all tubes.

3. Add 1ml of 4mM PNPP to all tubes.
4. Place tubes in the indicated (Fig. 8) water baths for 3 minutes to allow them to come up to temperature. Note that they are in pairs. The reason for this is that each incubation temperature requires its own separate blank.
5. While tubes are still in their water baths, add 1ml of enzyme solution, or 1ml of water as indicated in Figure 8. Be sure to note the exact time of the addition for each tube in Table 5. The tube receiving the water will serve as the blank for that particular temperature.
6. After exactly 15 minutes incubation, remove each tube and immediately add 3 ml of the NaOH solution.
7. Be sure the spectrophotometer is turned on and had at least a 10 minute warm-up. Set the wavelength to 410 nm.
8. Pour the contents of tubes #1 and #2 into cuvettes. Use the contents of tube #1 as a blank to zero the spectrophotometer.
9. Measure the Absorbance of the cuvette with tube #2's contents and record in Table 5. Then empty both cuvettes back into their respective tubes, rinse with deionized water and dry with a Kimwipe, and then add the contents of tubes #3 and #4 to each.
10. Use the contents tube #3 as a blank to zero the spectrophotometer. Then measure and record the Absorbance of the cuvette with tube #4's contents. Measure the Absorbance of the remaining tubes in the same way. The cuvettes need to be rinsed between measurements because the concentration of PNP does not necessarily increase with each pair.
11. Remember, the spectrophotometer must be reset to zero for each pair of tubes! In other words, every odd numbered tube will serve as a blank for the next even numbered tube.
12. Take the absorbance readings to your Standard Curve and convert to mM/min PNP formed and record in Table 5.
13. Calculate the rate of PNP formation by dividing the mM/ml PNP formed by 15 min. (the incubation time). Record in Table 5.

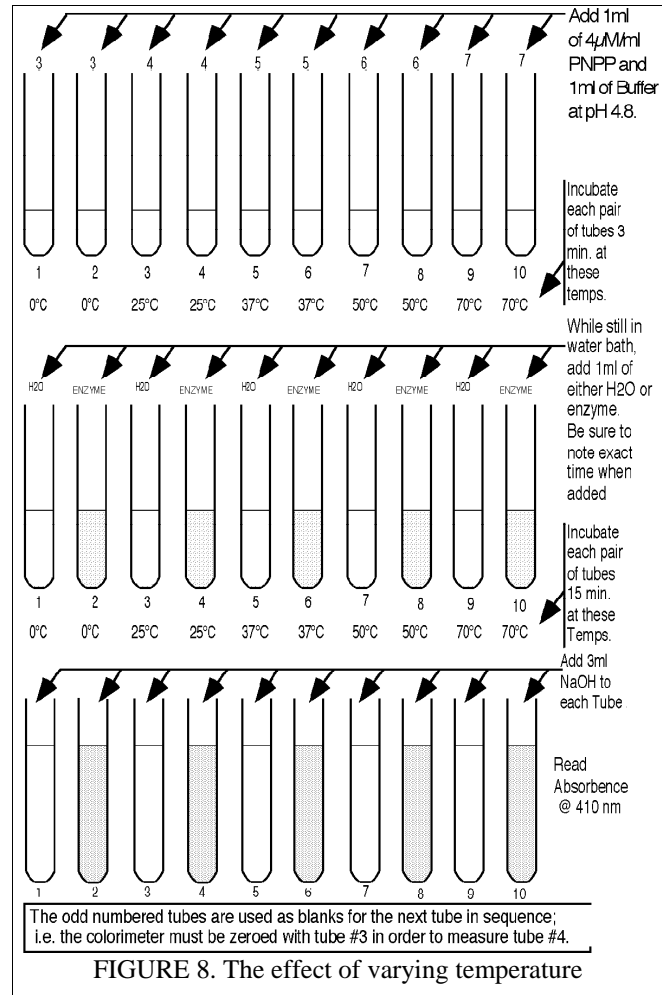


Table 5. Effect of Temperature						
Tube #	Start time	Stop time	Temp.	Abs.	mM PNP formed	Rate mM/min
1			0°C	0		
2			0°C			
3			25°C	0		
4			25°C			
5			37°C	0		
6			37°C			
7			50°C	0		
8			50°C			
9			70°C	0		
10			70°C			

F. ANALYSIS

One of the key ways to analyze and compare enzyme catalyzed reactions is to present the data graphically. By graphing the amount of PNP formed against time (Table 2), you can see what the reaction does over time. To study the effects of substrate concentration, pH, and temperature, the best graphic presentation is one which plots the rate of the reaction against the variable concerned (sub. conc., pH, temp.). Recall that the rates were calculated in mM PNP formed per minute by reading the amount formed in Tables 3-5, and dividing by 15 minutes (the incubation time in all cases). For example, to graph the results of the substrate concentration experiment (Table 3), plot the substrate concentration on the X axis and the corresponding rates on the Y axis. The data for the temperature and pH sections may be plotted in a similar manner. Do not forget to use trend lines for your graphs.

1. Create your Standard Curve from your data in Table 1. Follow directions from the previous lab to create the spreadsheet and graph in excel. Find a formula for your curve so that you can calculate concentrations for your experiments below.
2. Create a graph of rate of reaction versus time. For your trend line, you may want to choose a polynomial one that best estimates the curve of the data points.
3. Graphs for substrate concentration, pH, and temperature should be done using rates. Again, use may use a polynomial trend line if it best fits your data.

QUESTIONS FOR YOUR REPORT

(2-Day Lab – 20 pts)

1. Present an abstract of your work. Your abstract should include a brief description (1/2 page) of 1) what you set out to learn, 2) what procedures you performed, and 3) a summary of your data or discoveries.
 2. Present your data for this exercise. Include all tables and graphs (include all units and labels!)
 3. Explain the process of how you used your data to calculate the amount of PNP formed and rates of reaction.
 4. Explain how time affected the rate of reaction. Also give a molecular explanation to your findings (E.g. How do the enzyme and substrate interact differently under varying conditions? How do their shapes change?).
 5. Explain how substrate concentration affected the rate of reaction. Also give a molecular explanation to your findings.
 6. Explain how pH affected the rate of reaction. Also give a molecular explanation to your findings.
 7. Explain how temperature affected the rate of reaction. Also give a molecular explanation to your findings.
 8. Some of your findings are probably different from what you predicted. Describe any differences and explain what you think might have caused the differences.
- Refer to your textbook for questions 9-10.*
9. Pretend that you are developing an enzymatic drug to cure Tay-Sachs disease, a lysosomal disorder. What should the optimal pH and temperature conditions be for your drug? Draw ideal rate of reaction graphs for each condition (rate vs. variable).
 10. Imagine that your drug catalyzes the breakdown of lipids that contain fatty acids. Choose a lipid containing fatty acids and write out a balanced reaction showing how the fatty acids are released.